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We find that tamoxifen is a potent activator of estrogen receptor (ER) mediated induction of promoters regulated by AP-1 sites. This contrasts with the inability of tamoxifen to activate otherwise identical promoter constructs bearing a classical ERE. Tamoxifen agonism at AP-1 sites is cell type specific occurring in cell lines of uterine origin but not of breast origin. It thus parallels tamoxifen agonism in vivo. AP-1 proteins such as Jun or Jun/Fos are needed for tamoxifen stimulation, which appears to increase the transcriptional efficiency of these proteins even when they are provided at optimal amounts. The DNA binding domain (DBD) of ER is required for tamoxifen activation at AP-1 sites whereas estrogen activation is mostly independent of this domain. This suggests the existence of two pathways of ER action at AP-1: an α (DBD dependent) pathway activated by tamoxifen, and a β (DBD independent) pathway activated only by estrogen. Fusing VP16 transcriptional activation functions to ER super-potentiates the β, but not the α, pathway. We discuss models for the two pathways and the implications of these results.

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# INTRODUCTION

A long standing puzzle of estrogen receptor biology concerns the paradoxical estrogen-like effects of tamoxifen and related antiestrogens. These agents, which are used to treat and possibly prevent hormone dependent breast cancer (1-3) bind to the estrogen receptor (ER) and block its activation by estrogen (4). Although tamoxifen is usually an antagonist of estrogen action, it sometimes behaves like estrogen. For example, whereas tamoxifen does not stimulate breast tissue growth, it does stimulate uterine growth and the transcription of many estrogen-regulated genes in uterus, including C3 component of complement, alkaline phosphatase, progesterone receptor, and IGF-I (5-8). These estrogen-like effects of tamoxifen also may be seen in patients. They include an increase in cervical hyperplasia (9, 10), an increased risk of uterine cancer (11), and an unpredictable stimulation of breast tumor growth during tumor progression (12, 13). These effects complicate the potential use of tamoxifen as a preventative for breast cancer.

The origin of tamoxifen agonism seems paradoxical, given our current understanding of ER action at classical estrogen response elements (EREs) (14). Estrogen binding allows ER to dissociate from a heat shock protein complex, dimerize, bind to EREs, and then stimulate transcription. Two transcriptional activation functions in the ER contribute to this process: AF-1 in the receptor amino terminal domain is hormone independent, whereas AF-2 in the C-terminal ligand bind domain (LBD) functions only when estrogen or another agonist is bound (15). One of the antiestrogens ICI 164,384 (ICI) blocks ER action because it inhibits ER dimerization (16), and thereby prevents high affinity interaction with EREs (17). ICI also reduces ER protein half life (18). Tamoxifen, in contrast, allows ER binding to the ERE but inhibits gene expression because it does not allow AF-2 to function (19). The tamoxifen bound ER therefore cannot stimulate transcription unless AF-1 is sufficiently strong to activate transcription by itself, and in most cell types AF-1 is too weak to do this. Only in certain cell types, such as chick embryo fibroblasts, does AF-1 has strong activity. Hence tamoxifen should have agonist activity only in those cells or

others in which the AF-1 becomes active. This expectation is fulfilled when tested with classical EREs (20). It is unclear, however, whether this AF-1 driven mechanism underlies tamoxifen agonism in vivo. Tamoxifen has potent estrogen-like effects in cells in which AF-1 is weak (for example, endometrial cells and cervical cells, see below). Furthermore, tamoxifen agonism sometimes exceeds estrogen induction in the uterus (5, 6). These observations are hard to reconcile with tamoxifen action at classical EREs.

What, then, is the origin of the estrogen-like effects of tamoxifen on, for example, the uterus? Recently, an alternative pathway of ER action has been reported (21-23) in which the receptor appears to be able to stimulate transcription from a transfected promoter that contains an AP-1 site, the cognate binding site for the transcription factors Jun and Fos, rather than an ERE. The mechanism by which the receptor stimulates transcription in this "AP-1 directed" pathway is unknown. It is believed to involve protein-protein, rather than protein-DNA contacts, because it is partly independent of the ER DNA binding domain. In this study we test the effects of antiestrogens on this alternate ER pathway. We show that antiestrogens are strong agonists of AP-1 dependent transcription and analyze the molecular requirements for tamoxifen agonism. We suggest that activation by this pathway is likely to underlie some of the unexplained agonist effects of tamoxifen in vivo.

# Body

## MATERIALS AND METHODS

### Plasmid Construction

All reporter genes in this study have been modified by digestion with EcoO109 and Nde1 to remove an AP-1 site in the backbone of pUC. Thus, Coll73 and Coll60 are formerly ΔColl73 and ΔColl60 (Lopez *et al.*, 1993). Coll73-LUC was constructed by cloning a BamHI/PvuII fragment, that spanned the luciferase transcription unit, from pMG3 (Brasier and Ron, 1992) into coll73, which had been digested with BamHI and SmaI to remove the CAT transcription unit. EREcoll60 and EREcoll73 was prepared by ligation of a consensus ERE (AGGTCACAGTGACCT), (52), into the HindIII site upstream of coll60 and coll73, respectively. [AP1]2coll60 contains an oligonucleotide, (AGCTTCCATCGTCCTGAGTCAGCA), doubly inserted in a head to tail orientation, at the HinDIII site of coll60, and was identified by sequence analysis of random clones. All other reporter genes have been previously described (25, 29).

Expression vectors for ER and ER mutants (15), VP16-ER (30), c-jun (53) and c-fos (54) have been described. For this study, all ER cDNAs were cloned into the EcoRI site of the SG5 expression vector (55). The VP16ER cDNA was also cloned into SG5, to form the vector VER, in two steps. The expression vector Vp16ER1-422 (30) was digested with SstI, repaired with T4 polymerase, and ligated to EcoRI linkers. The resulting EcoRI fragment was sub-cloned at the equivalent site of SG5 to generate the vector VER1-422. This was digested to completion with HindIII and BglII and the equivalent (HindIII/BamHI) fragment from HE0 was replaced at this location. VERΔDBD was constructed by substituting a Not1/BglII fragment from HE11 (cloned in SG5) into VER digested with the same enzymes.

The GST wild type ER fusion gene (GST-HEG0) was constructed by ligation of the EcoRI fragment from pSG5-HEG0, spanning the ER cDNA, into pGEX5X-1, one of the vectors of the

pGEX series (Pharmacia Biotech Inc., Piscataway, N.J., USA). GST-hELBD was constructed in two steps. An Xba fragment from HE19G was inserted into the equivalent position of XbaI digested SG5-HE14, which spans the ER LBD (Kumar *et al.*, 1987). Then, an EcoRI fragment spanning this ER cDNA was cloned into pGEX-3X. To prepare GST-hEN185 an EcoRI/KpnI fragment spanning the ER amino terminus was obtained from the vector EGE (15), repaired and cloned into pGEX-5X-1 digested with SmaI and EcoRI.

**Tissue Culture and Transfections**

Cells were maintained and transfected as previously described (25). Hormones were added two hours after plating in the following concentrations, estradiol 100nM, ICI 164,384 1μM, hydroxy-tamoxifen 1μM, tamoxifen 5μM. F9 cells were seeded at 30% confluence upon 1.5cm dishes and transfected overnight by calcium phosphate coprecipitation with 5μg reporter gene, 1μg actin β-HCG, and 1μg of HEO, 300ng each of c-jun and c-fos expression vectors. The cells were glycerol shocked and refed in growth medium containing hormone or ethanolic vehicle. In transient transfections, optimal amounts of HE0 were employed and were as follows:- CHO (100ng); HeLa (1μg); NIH3T3 (1μg); HepG2 (1μg); SHM (300ng); SY5Y (300ng); CEF (100ng); CV-1 (3μg); MDA453 (3μg); F9 (1μg) and Rat2 (300ng).

CAT assays were carried out as previously described (25), except that the cells were harvested one to two days after transfection instead of three. CAT activities were defined as the increase in cpm per hour at room temperature (corrected for background) per 100μl of cell extract, normalized to production of 100 standard units of βHCG, from a cotransfected reporter gene, actin-HCG. Luciferase assays (56) were performed on similar extracts that were used for CAT assays. Light units were defined as the luciferase activity per 100μl of cell extract per 100 standard units of βHCG. Luciferase activities in Tables I and II obtained in the absence of ER and hormone were set at 1 unit. Results, from each cell line or ER mutant, were then calculated relative to this value.

### GST Fusion Protein Binding Assay and *in vitro* Translation

Procedures were carried out as previously described (29).

## Results

### Both Estrogen and Antiestrogens Activate Transcription of the Human Collagenase Promoter Containing an Intact AP-1 Site

To examine the effects of antiestrogens on the AP-1 directed pathway, we transfected reporter genes derived from the human collagenase promoter, in which a consensus AP-1 site is located between -60 and -73 base pairs from the start of transcription (Fig. 1, (24)). Both estrogen and antiestrogens activated the collagenase promoter (coll517, Fig. 1A) in HeLa cells in the presence of transiently expressed human ER. In these cells tamoxifen was more potent an activator than estrogen. This pattern was retained with shorter versions of the promoter that have the AP-1 site (coll73), but was lost when the AP-1 site was deleted (coll60) or was inactivated by point mutations (coll517mAP1). None of these responses were obtained in the absence of transfected ER (data not shown). In derivatives of CHO cells that stably express high levels of the human ER (ERC1 cells, (25)), the coll73 promoter, containing the AP-1 site, was again activated by both estrogen and antiestrogens (Fig. 1B). The response of the collagenase promoter to increasing dosage of ligand (Fig. 1C) indicates that the half maximal dose for hydroxy tamoxifen and ICI is about 10 times, and tamoxifen 100 times, that for estrogen. This is consistent with the known binding affinities of these compounds to the estrogen binding site on the receptor and suggests that they are stimulating transcription through that site.

We conclude that antiestrogens are agonists at the collagenase promoter, that the AP-1 site is required for this activity, and that antiestrogens are working through the ER.

### Classical EREs Do Not Substitute for AP-1 Sites in Antiestrogen Activation

We next compared the activity of antiestrogens in the AP-1 pathway with their activity in the ERE directed, or classical, pathway. Direct substitution of an ERE for the collagenase AP-1 site (ERE-coll60, Fig. 2A), restored estrogen response to the core collagenase promoter, but not antiestrogen response or the basal activity associated with the AP-1 site. Thus, a classical ERE cannot substitute for the AP-1 site, indicating that the AP-1 site has a unique function in activation by antiestrogens. We also tested the effect of combinations of elements upstream of the collagenase core promoter. A promoter with both an ERE and an AP-1 site (ERE-coll73) gave a large estrogen response, and retained responses to antiestrogens. Substitution of twin AP-1 sites upstream of the core collagenase promoter ([AP1]2-coll60) conferred an estrogen response equal to that of an ERE plus an AP-1 site, and a tamoxifen response that was nearly as great as the response to estrogen. Thus the strength of antiestrogen response is a direct function of the presence and number of AP-1 sites.

When the collagenase AP-1 site was placed upstream of the herpes virus tk promoter both antiestrogens and estrogens were able to activate transcription, although this response was not as robust as with the native collagenase promoter (Fig. 2B). This may suggest that other features of the collagenase promoter, besides the AP-1 site, contribute to the ability of estrogen and antiestrogens to activate. A control reporter regulated by a classical ERE (ERE-tk, Fig. 2B) was activated by estrogen, but not by antiestrogens. Similar responses to that shown here in CHO derivatives occur in HeLa cells (not shown, (20)). We conclude that an AP-1 site, but not an ERE, can confer antiestrogen inducibility on linked promoters.

## Antiestrogens Are Agonists of the AP-1 Pathway in Many Cell Types Including Endometrial Cells, But Not in Breast Cells

To test whether the AP-1 pathway was widespread, we examined collagenase promoter expression in a range of cell lines representative of different tissue types. Each cell line was transfected with coll73-LUC and a human ER expression vector. Table I shows that both estrogen and antiestrogens activated the collagenase promoter in most cell types. This response occurred with

cell lines derived from ovary, cervix, liver, myometrium, neuroblastoma, and kidney. Only two cell lines, including F9 cells (which are investigated below), were not activated by any ligand. Thus, antiestrogen agonist effects occur in cells of diverse origin.

One line, MDA453 breast cancer cells, allowed potent activation by estrogen but not by tamoxifen (Table I). This suggests that antiestrogen action at the collagenase promoter might have a cell specific component. To further explore this phenomenon, and to test whether similar hormone effects could be detected at physiological levels of ER, we examined expression of the collagenase promoter in cells that express endogenous ER. We chose Ishikawa cells, an endometrial cell line that is believed to provide a model of tamoxifen agonism on the uterus, and two breast cancer cell lines, MCF-7 and ZR75, both of which are known to respond to estrogen but not tamoxifen. In Ishikawa (Fig. 3A) the collagenase promoter was activated by estrogen and tamoxifen, but the ICI compound was usually inactive in the presence of endogenous receptor (activity occurred once in five experiments, not shown). This parallels the reported potency of tamoxifen and ICI on cell growth and induction of progesterone receptors in these cells (6). When receptor levels are raised by transfection ICI becomes more potent. In MCF-7 cells, estrogen, but not tamoxifen, activated the collagenase promoter (Fig. 3B). The same pattern occurred in ZR75, and could be seen more clearly when extra receptors were supplied by transfection. Again, this parallels the reported absence of tamoxifen effects on cell proliferation and gene expression in breast cancer cell lines (22, 26). We conclude that tamoxifen activates the collagenase promoter in cells with physiological levels of ER, but that the response shows tissue restrictions. Tamoxifen activity occurs in endometrial cells, but not in breast cells, and thus parallels the known range of tamoxifen agonism.

## AP-1 Proteins Are Required for ER Action at the Collagenase Promoter

To test whether AP-1 proteins, as well as their cognate binding site, were required for the AP-1 pathway, we examined the effect of Jun and Fos overexpression on the hormone response of the collagenase promoter. Although HeLa cells in the presence of ER but the absence of Jun/Fos

overexpression allow antiestrogens and estrogens to activate through the AP-1 site, the induction by ligands is greatly increased in the presence of overexpressed Jun (Fig. 4A) or Jun/Fos. Fos overexpression had little effect in the absence of transfected Jun. These observations suggest that Jun homodimers or Jun/Fos heterodimers occupying the AP-1 site contribute to the ability of ER to activate transcription in the AP-1 directed pathway. To confirm that AP-1 proteins are needed we turned to F9 cells, which have only low levels of endogenous AP-1 (27, 28). Transfection of an expression vector for estrogen receptor into these cells did not support hormone activation of the collagenase promoter (Fig.4B, see also Table I), whereas it gave estrogen activation at an ERE (not shown). This suggests that AP-1 proteins are needed for hormone activation of the collagenase promoter. Cotransfection of ER with Jun/Fos restored induction by both estrogen and antiestrogens in F9 cells, albeit at lower levels than that seen in HeLa cells. In addition there was some activation by unliganded ER, an effect similar to one that we have previously observed with the thyroid hormone receptor (29). Thus, the inability of F9 cells to allow a hormone response at the collagenase promoter can be overcome with AP-1 supplied by transfection. We conclude that hormone effects at the AP-1 site require AP-1 protein. However, the dramatic difference between the hormone response of HeLa and F9 cells when both are supplied with Jun and Fos indicates that cell specific factors in addition to AP-1 abundance regulate the strength of the AP-1 directed ER pathway. We investigate the extent of the cell specificity below.

In these studies of F9 cells we determined the amounts of AP-1 required for optimal collagenase promoter activity. Fig.4C shows that Jun, Fos, and a combination of both, increased basal activity of the collagenase promoter (in the absence of ER) which reached a maximum with 300ng of expression vector. These optimal amounts were employed in the cotransfections with ER (Fig. 4B). ER activation at AP-1 sites therefore appears to increase the transcriptional efficiency of Jun and Fos even when they are provided at optimal amounts. It is therefore unlikely that ER dependent activation at AP-1 sites is due to changes in the amount of AP-1 (see Discussion).

## ER Binds Jun But Not Fos *in vitro*

To test whether ER effects upon AP-1 might reflect direct biochemical interaction between the ER and AP-1 proteins, we examined whether they specifically interact in solution. As shown in Fig. 5A, an estrogen receptor protein fused to glutathione S-transferase (ER-GST), and attached to agarose beads, pelleted in vitro translated Jun from solution (lane 3), whereas a control GST protein did not (lane 2). Similar binding occurred with the ER amino terminal domain (compare lanes 7 and 8), but not with the LBD (lanes 5 and 6). Neither the intact ER nor its isolated domains bound Fos (Fig. 5B). These results indicate that Jun, but not Fos, binds ER in vitro, and that a major target of Jun is the ER amino terminus.

## Tamoxifen Activation at AP-1 Requires the ER DBD, Whereas Estrogen Activation is DBD Independent in Some Cell Types.

We next examined which domains of the ER mediate hormone action. We introduced truncated derivatives of the ER into HeLa, CHO, and MDA453 cells and examined their ability to activate a reporter genes driven by the collagenase promoter with its AP-1 site (Fig. 6, left side) or a reporter gene driven by control promoter with an ERE (Fig. 6, right side). Deletion of the DNA binding domain (DBD) completely eliminated estrogen activation at an ERE in all three cell types (HE11, Fig. 6). Deletion of the DNA binding domain also eliminated tamoxifen activation at AP-1 sites, whether the substantial tamoxifen activation in HeLa and CHO cells, or the marginal amount in MDA cells. In contrast, removal of the DBD did not abolish estrogen activation at the AP-1 site in any of the cell lines. Indeed, estrogen activation at the AP-1 site in CHO cells was equally strong with or without the ER DBD. This is consistent with previous observations that estrogen response at AP-1 sites shows independence of DNA binding in CEF (23). Thus, the requirement for the ER DBD varies according to the ligand. We suggest below (Discussion) that the differential requirements for the ER DBD may indicate the existence of more than one pathway of ER action at AP-1 sites.

The ER amino terminus also plays an important role in activation at the AP-1 site. Deletion of the amino terminus (HE19) abolishes tamoxifen activation in both HeLa and CHO cells. Some

estrogen activation remains in these cells whereas it is lost in MDA cells. Control measurements with the ERE regulated reporter indicate that the amino terminal deleted receptor is active in all three cell lines. Thus the requirement for the amino terminus is also partly ligand dependent.

Deletion of the ligand binding domain leaving the amino terminus and DBD, (HE15) gives a constitutively active receptor able to activate at an ERE in all three cell lines. Although this receptor is inactive in MDA cells and weak in CHO cells, it is a potent activator at the AP-1 site in HeLa cells. Thus the requirement for this domain for AP-1 activation shows cell type specificity. This again suggests that activation through AP-1 may occur by more than one mechanism.

## ER Can Target an Exogenous Transactivation Domain to the Collagenase Promoter, Independently of the ER DBD

One possible mechanism for ER activation at AP-1 sites is that the receptor might directly bind to the AP-1 complex at the promoter (see Discussion) and from there influence transcription. A prediction of this model is that ER should be able to target heterologous transcriptional activation functions to an AP-1 regulated promoter.

In order to test this proposition, we examined the effects of linking the strong VP16 transcriptional activation domain to the amino terminus of the ER (V-ER). To monitor activity we used a luciferase reporter gene regulated by an AP-1 site and CAT reporter gene driven by an otherwise identical promoter with an ERE. The V-ER chimaeric receptor gave markedly enhanced activation at an ERE in CHO cells (Fig. 7A). It was activated both by estrogen and antiestrogens reflecting the ability of VP16 to override the need for AF-2 and consistent with previous reports for this "super-receptor" (30). The V-ER receptor gave equally enhanced activation at the AP-1 site (Fig. 7A). Since the domain studies in CHO cells had indicated that estrogen activation at the AP-1 site was completely independent of the DBD, we also tested a version of the super-receptor in which the DBD was deleted (VERΔDBD). This receptor, as expected, failed to activate at an ERE. It was, however, a potent activator at the AP-1 site (Fig. 7A). A control fusion of the VP16 domain to the yeast GAL4 DNA binding domain did not increase collagenase promoter

transcription. Thus, the superactivation by VP16 is dependent upon sequences in the ER protein. These observations indicate that super-receptors are super-activators at AP-1 sites in CHO cells and in a DBD-independent manner.

The super-receptors had qualitatively similar properties in HeLa cells, although the potentiation by the VP16 domain at both types of response element was less dramatic (Fig. 7B). Again, however, estrogen activation at the AP-1 site was increased by the super-receptor and in a DBD-independent manner. There was one notable lack of response to the super-receptor. Tamoxifen activation with the full length ER was hardly increased by fusion to VP16 (Fig. 7B). This suggests that there may be one pathway of activation at AP-1 sites that does not respond to exogenous transcriptional activation functions.

## An Exogenous Transcriptional Activation Domain Potentiates the Action of an ER Without an LBD at an ERE, But Not at an AP-1 Site.

We had observed in our domain studies that an ER without the LBD was a potent constitutive activator of the AP-1 pathway in HeLa cells. To explore whether transcriptional activation functions were involved in this pathway, we examined the effects of fusing the VP16 activation domain to this receptor (V-ERΔLBD, Fig. 8). The presence of the VP16 domain greatly potentiated transcription from an ERE at every level of expression (Fig. 8B), but failed entirely to potentiate transcription activation by ER from the AP-1 site. Thus the activation pathway of the LBD deleted receptor at AP-1 sites appears not to respond to exogenous transcriptional activation functions. We argue below that this suggests the existence of an ER pathway that activates transcription from AP-1 sites independent of ER associated transcriptional activation functions.

## Conclusions

The observations described above show that the estrogen receptor can stimulate transcription of the collagenase promoter through a non-classical pathway mediated by the AP-1 site. Both estradiol and the antiestrogens tamoxifen, 4-hydroxy-tamoxifen, and ICI 164,384 activate transcription in this AP-1 directed ER pathway, whereas only estrogen activates transcription at classical EREs. The AP-1 directed response is relatively common and active at physiological ER levels. Our results thus confirm previous observations that ER can activate AP-1 responsive genes (21-23) and suggest that AP-1 directed responses may be more widespread than hitherto suspected. Other groups have previously reported that ER inhibits AP-1 activity (31-33). We find that the estrogen response at AP-1 sites is biphasic and high levels of ER can squelch the response (see Fig. 3B, Fig.4A, data not shown). Thus, ER can stimulate or repress collagenase expression.

It has not been previously reported that antiestrogens are agonists of AP-1 dependent transcription. Other groups who examined the effects of antiestrogens on AP-1 driven reporter genes restricted their studies to breast cells and found that tamoxifen had no measurable effects (Philips *et al.* 1993). Our studies also show that antiestrogens have no effects on AP-1 dependent transcription in breast cells. Indeed, tamoxifen and other antiestrogens activate the AP-1 directed ER pathway only in restricted cell types. This is discussed further below. This is also the first report that the antiestrogen ICI, which has previously been considered to a pure antagonist of estrogen action (34), may induce AP-1 responsive genes. ICI effects may have gone largely unnoticed because genes with classical EREs had been used to detect antiestrogen effects. Furthermore, optimal ICI induction of the collagenase promoter requires higher amounts of ER than induction by tamoxifen (Webb *et al.*, manuscript in preparation), and did not occur at physiological ER levels. This probably reflects the ability of ICI to lower the amount of ER to inactive levels (18), and may mean that ICI will show little agonism in vivo.

## Possible Mechanisms of ER Activation at AP-1 Sites

How does ER activate transcription via AP-1 sites? In classical estrogen response, ER activates transcription by binding to EREs in the promoters of estrogen responsive genes. Activation at the collagenase AP-1 site must involve an unusual mechanism because ER does not bind this promoter (35). More evidence for a novel pathway comes from the ability of ICI, a compound which interferes with ER dimerization (16) and high affinity interaction with EREs (17), to activate the response. Furthermore, activation at an AP-1 site requires Jun protein, and, unlike activation at an ERE, does not absolutely depend on an intact ER DNA binding domain. The likeliest explanation for these observations is that ER activates transcription at AP-1 sites via protein-protein interactions.

It is possible, in principle, that the ER induces synthesis of AP-1 proteins via protein-protein interactions and thereby indirectly activates the collagenase promoter. We believe, however, that this type of mechanism is unlikely because induction still occurs when AP-1 proteins are provided at optimal levels from transfected expression vectors. In agreement with this conclusion investigators who have observed ER activation at AP-1 sites in MCF7 cells and HepG2 cells also found that AP-1 levels were unaltered by estrogen in conditions where estrogen activated at an AP-1 site (21, 22).

These observations are more consistent with direct ER activation at AP-1 sites. We suggest that this may occur by two alternative mechanisms. This idea stems from observations of the variable requirement for the ER DNA binding domain (DBD). The DBD is dispensable for estrogen action at AP-1 elements in CHO cells and at least partly dispensable in HeLa and MDA453. Similarly the DBD is not absolutely required for estrogen activation of the AP-1 pathway in chicken embryo fibroblast cells (CEF, (23)). In both HeLa and CHO the DBD is required for induction by tamoxifen. We therefore propose the existence of both DBD dependent and DBD independent pathways of ER activation through AP-1. The DBD dependent, or $\alpha$, pathway is activated by tamoxifen (and possibly by estrogen) and predominates in HeLa and Ishikawa cells. The DBD independent, or $\beta$, pathway is activated only by estrogen. The $\beta$

pathway predominates in breast cancer cells. Both pathways may be operative in CHO cells because estrogen and tamoxifen induction show different requirements for the ER DBD.

The nature of the tamoxifen stimulated α pathway is perhaps most difficult to unravel. In addition to the ER DBD the amino terminal domain is essential for tamoxifen agonism (Fig.6; Table II) at AP-1 sites. At classical EREs tamoxifen agonism also requires the amino terminus and reflects the activity of AF-1 (20). For several reasons, however, we think that the involvement of the ER amino terminus in the α pathway is unlikely to reflect transcriptional activation by the ER AF-1 function. First, tamoxifen agonism is strong in cells in which AF-1 function is weak (e.g. HeLa, Fig. 6B), and weak in cells in which AF-1 function is strong (e.g. MDA453). Second, our preliminary studies in HeLa cells indicate that point mutations affecting the ER AF-1 function do not reduce the activity of ER at AP-1 sites (Webb *et al.*, in preparation). In addition, studies with the activation domain from VP16 fused to various ER derivatives indicate that this potent exogenous activator is unable to potentiate tamoxifen activation at AP-1 sites in HeLa cells and unable to potentiate the ligand independent activity of an ER without an LBD at an AP-1 site. In sum these observations suggest that transcriptional activation functions associated with the ER are not involved in the AP-1 α pathway.

We propose instead that in the α pathway the ER interacts with some unidentified target protein and sets off a cascade leading to increased Jun/Fos transcriptional activity (Fig. 9). Presumably the amino terminus and DBD are needed for this hypothetical protein interaction. In these interactions the ER could affect any of the mechanisms that regulate AP-1 DNA binding or post binding transcriptional activity. It could sequester one of the inhibitors of Jun activity (36-39), or increase DNA binding of the AP-1 complex through putative DNA binding stimulatory factors (40) or the Ref-1 activator of AP-1 binding (41). Our preliminary studies with extracts of tamoxifen treated cells suggest, however, that there is no significant increase in AP-1 binding activity after tamoxifen treatment (Webb et al. unpublished). A more likely possibility is that the ER could enhance the activity of signal transduction pathways that increase the transcriptional potency of Jun, such as that through the JNK1 Kinase (42). It has recently been reported that

estrogens and antiestrogens can activate adenylate cyclase (43), which is known in some cells to lead to activation of Jun/Fos (44), and it is possible that the α pathway shares steps with this process.

We suggest that the β pathway involves direct action of ER at AP-1 regulated promoters where it is tethered by protein interactions that replace DNA binding (Fig.9). This may involve direct interaction with Jun and synergism between ER activation domains and those of the AP-1 proteins. The most telling observation in favor of this model is that ER can target the VP16 activation domain to promoters with an AP-1 site. Such targeting occurs to some degree in all cell types, as does the β pathway. This provides evidence that the ER interacts functionally with such promoters in vivo. VP16 targeting persists in the absence of the ER DBD, linking it to the β pathway.

## Physiological Significance of Antiestrogen Activation Through AP-1 Sites

In this paper we have presented evidence that antiestrogens act as agonists of AP-1 dependent collagenase promoter transcription in a cell type specific pattern. This suggests the intriguing possibility that the estrogen-like effect of tamoxifen in vivo on certain tissues reflects the presence of AP-1 sites in key target genes that control growth and differentiation along with a tamoxifen stimulated AP-1 activation pathway. Tamoxifen induces a wide range of genes in Ishikawa cells and other uterine cells, which are thought to model tamoxifen agonism on the uterine endometrium. These include those for the C3 component of complement, progesterone receptors, alkaline phosphatase (5, 6, 8), and the IGF-I gene, a pivotal gene in uterine growth (7). Tamoxifen also stimulates the growth of Ishikawa endometrial tumor cells in a serum dependent manner (45) and the growth of human endometrial tumors in nude mice (26, 46). In our studies we found that tamoxifen activates the AP-1-regulated collagenase promoter in Ishikawa cells. Tamoxifen is also a potent activator of the AP-1 directed pathway in HeLa cells, which are of cervical origin, another tissue on which tamoxifen has estrogen-like effects. Although tamoxifen has partial agonist activity on ERE regulated promoters in some cells such as CEF (20) and breast cells (Fig. 6),

tamoxifen has limited agonist activity at classical EREs in HeLa and other uterine cells (Figs. 3, 6). We therefore propose that many of the genes controlling the hormone induced growth and differentiation of the uterus may be regulated by AP-1 sites and that tamoxifen bound ER can efficiently activate transcription of those genes. Indeed, we predict that the recently reported tamoxifen activation of IGF-I in the uterus Huynh, 1993 #239] is, like the effect of estrogen in HepG2 cells (21), mediated by the AP-1 site in the promoter.

Estrogen-like effects of antiestrogens have been reported in a number of other tissues including the brain (for example, see (47)). We suggest that this reflects the common occurrence of AP-1 sites in the promoter regions of target genes and of an active AP-1 directed ER pathway in brain. The vasopressin gene and the brain creatine kinase gene, for example, each have several AP-1 sites, no ERE, and are induced by estrogen and antiestrogens ((48, 49), D. Dorsa personal communication). Other instances of antiestrogen agonism such as prolactin in GH4 pituitary cells (50) and c-myc in HeLa cells (51), may be due to activation of unrecognized AP-1 sites in the promoters of these genes.

In contrast to the estrogen-like effects of tamoxifen on the uterus, tamoxifen and other antiestrogens are without agonist activity on breast tissue. This has been documented both in vivo and with breast cancer cell lines in which antiestrogens fail to activate gene expression, or growth either in culture or in nude mice (22). In parallel to this we found that tamoxifen and other antiestrogens do not induce the AP-1 site containing collagenase promoter in breast cancer cell lines (MDA453, MCF-7 and ZR-75). These cells do allow activation of AP-1 site containing promoters by estrogen. It remains possible that AP-1 directed antiestrogen agonist effects could still be important in breast tumor progression. Some patients who fail to respond to tamoxifen therapy show a favorable response to tamoxifen withdrawal , suggesting that tamoxifen may have become an agonist of tumor growth (12, 13). The emergence of tamoxifen agonism at AP-1 sites might underlie this process.

In summary, the major estrogen effects on tissue growth and differentiation may be exerted through the action of ER on gene expression through AP-1 sites rather than through classical

EREs. We suggest that future analysis of estrogen action should consider both classical and AP-1 directed responses. We also propose that screening for therapeutic antiestrogens should use both classical and AP-1 regulated reporter genes. In this way it should be possible to identify antagonists free from potentially undesirable agonist properties and new mixed agonists/antagonists for preventative therapy.

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# APPENDIX

## Figure Legends

Table 1

**Fig.1.** Estrogen and Antiestrogens Stimulate Expression of the Collagenase Promoter with an Intact AP-1 Site.

**(A)** CAT assays of HeLa cells transfected with the indicated reporter genes and 3μg of human ER expression vector. Representations, at left, show the human collagenase promoter (shaded) and the consensus AP-1 site. CAT activities were from cells maintained in the absence of hormone or saturating concentrations of ICI 164,384 (ICI, 1μM), tamoxifen (5μM) or estradiol (100nM). CAT activity is normalized to a transfection control with the actin promoter driving expression of βHCG. Single representative experiments are shown, error bars represent standard deviation of triplicate hormone treatments. **(B)** Concentration dependence of estrogen and antiestrogen induction of coll73 in HeLa cells.

**Fig.2.** Classical EREs Do Not Substitute for AP-1 Sites in Allowing Antiestrogen Agonism.

**(A)** CAT assays from ERC1 cells transfected with reporters whose structure is shown on the left. ERE-coll60 consists of the consensus ERE from the vitellogenin A2 gene cloned upstream of the coll60 promoter. ERE-coll73 consists of the ERE upstream of the coll73 promoter. $[AP1]_2$-coll60 consists of a double insertion of a sequence overlapping the collagenase AP-1 site (from -73 to -52) upstream of the coll60 promoter. **(B)** CAT assays from ERC1 cells transfected with reporter genes based upon the herpes simplex virus TK promoter. ERE-TK-CAT consists of the ERE, fused to the TK promoter from -109 to +45 relative to the start site of transcription. AP1-TK-CAT consists of sequences overlapping the collagenase AP-1 site (from -73 to -52) upstream of the TK promoter. Averages of three to five experiments are presented.

**Fig.3.** Tamoxifen is an Agonist in Endometrial But Not in Breast Cells.

**(A)** Response of the transfected collCAT reporter in Ishikawa cells treated with estrogen or antiestrogen as in Fig.1. Left panel, collCAT response with endogenous ER. Middle panel collCAT response with 3 μg co-transfected expression vector for human ER. Right panel, response of an ERE regulated reporter. Averages of three individual experiments are shown. **(B)** Activity of the collagenase promoter in breast cell lines with endogenous ER. Left panel shows response of the collagenase promoter driving CAT in MCF7 cells (average of four experiments). Right panel shows activity of the coll promoter driving luciferase expression in ZR75 cells, either without or with 300ng of human ER expression vector. A single representative experiment with triplicate hormone treatments is shown.

**Fig.4.** Hormone Response at the AP-1 Site Requires AP-1 Proteins.

**(A)** Potentiation of hormone responses in HeLa cells by Jun or Fos. Cells were transfected with 5μg coll73-LUC and 1μg Actin-βHCG. Luciferase activities, normalized to HCG production, are presented. Transfections included, as indicated, 1μg of expression vector for ER, and 300ng vector for Jun or Fos. DNA was equalized with control expression vector. Hormone concentrations were as in previous experiments. **(B)** Effects of ER with and without transfected Jun and Fos on hormone induction of the collagenase promoter in F9 cells. Averages of five or six individual transfections are shown. **(C)** Response of the collagenase promoter to increasing amounts of Jun, Fos, or their combination, in the absence of ER. Transfections included up to 10μg of Jun or Fos expression vector and were normalized for DNA content.

**Fig.5.** The ER and Jun Interact in Solution.

**(A)** Binding of labelled Jun protein obtained with ER fused to GST (GST-ER). Schematic representations of ER fusions are shown above the panel. Autoradiograms of in vitro translated labelled proteins separated by SDS-Polyacrylamide gels electrophoresis after GST-agarose bead binding assays are shown below. The positions of size markers run in parallel are

noted. Lanes 1 and 4 represent 10% of the radiolabelled input Jun that was used in the remaining lanes. Lanes 2, and 3 are comparisons of binding of control GST beads and equimolar amounts of GST fused to full length ER (GST-HEG0). Lanes 5 and 6 compare control and GST fused to the ER ligand binding domain (GST-hELBD). Lane 7 and 8 compare control and GST fused to the ER amino terminus (GST-hEN185). **(B)** Results obtained with radiolabelled Fos. The arrangement of the lanes was the same as panel A. In both A and B autoradiographs were overexposed to reveal background binding by control GST beads.

**Fig.6.** The DNA Binding Domain of ER is Required for Tamoxifen Induction at an AP-1 Site, but Not Required for Estrogen Induction.

Reporters regulated by an AP-1 site (left panels), or an ERE (right panels) were introduced into Hela **(A)**, CHO **(B)**, or MDA453 cells **(C)**, with 100ng of expression vector for the ER derivative whose structure is indicated. The DNA binding domain is indicated with the striped box, the ligand binding domain (AF2) and the amino terminal (AF1) activation functions are marked. Cells were incubated with hormones, as in Fig.1A

**Fig.7.** Fusing an Exogenous Transactivation Function to the ER Increases Activation at AP-1 Sites.

A luciferase reporter regulated by an AP-1 site (left panels) and a CAT reporter regulated by an ERE (right panels) were introduced into Hela **(A)**, CHO **(B)** with expression vector for the ER derivative whose structure is indicated. CAT or Luciferase activities, normalized to HCG production are shown. Activator plasmids, are shown schematically at the left of the figure. ER domains are represented, as in Fig.6. The VP16 transactivation domain is represented as an oval. The GAL4 DNA binding domain is marked. 300ng of expression vector was used for CHO cells and 5μg for HeLa as these levels were optimal for VER dependent hormone induction at an ERE.

**Fig. 8.** Fusing an Exogenous Transactivation Function to an ER Derivative Without the Ligand Binding Domain Potentiates Gene Expression Mediated by an ERE but not by an AP-1 site.

A luciferase reporter regulated by an AP-1 site (left panels) and a CAT reporter regulated by an ERE (right panels) were introduced into HeLa cells with expression vector for ER derivatives. CAT or Luciferase activities, normalized to HCG production, are shown. Activator plasmids were cotransfected in increasing amounts and their structures are shown schematically at the left of the figure. HE15 (solid lines) lacks the LBD. V302C (dashed lines) is otherwise identical but has the VP16 activation domain at its amino terminus.

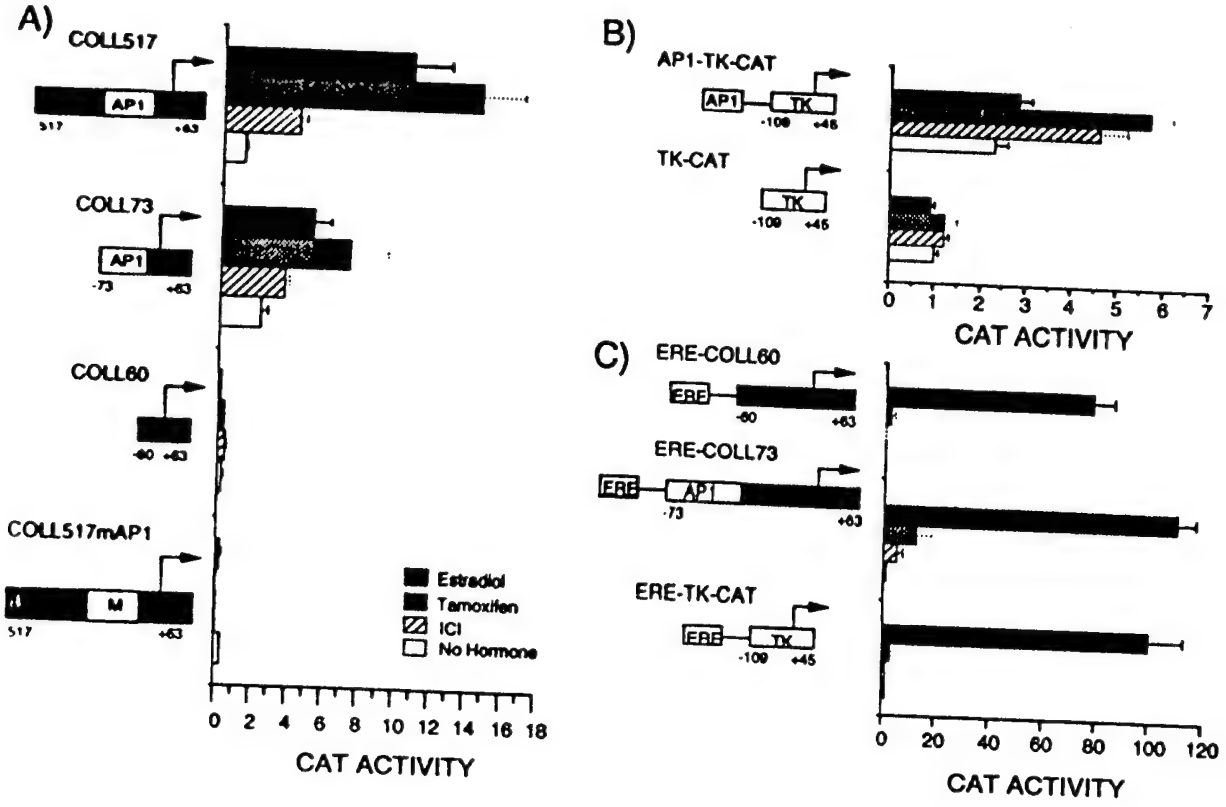
**Fig. 9.** A model of pathways of ER action. We propose that the ER can stimulate promoter activity either by the classical pathway (left) in which the receptor binds to EREs in the promoter region of target genes or by either of two pathways leading to activation of genes regulated by AP-1 sites. In the AP-1 $\alpha$ pathway, which requires the receptor DNA binding domain and is activated by tamoxifen (middle), ER interacts with an unknown target protein activating a cascade that increases the transcriptional efficiency of Jun/Fos at their cognate AP-1 site. In the AP-1 $\beta$ pathway, which is independent of the receptor DNA binding domain and is activated only by estrogen (right), ER is tethered to the AP-1 site by interactions with Jun/Fos.

| Cell Line | Origin | Coll73 Luciferase Activity (*1) | | | |
|---|---|---|---|---|---|
| | | No Hormone | ICI | Tam | E2 |
| HELA | CERVIX | 1.0 | 3.7 | 7.4 | 3.4 |
| NIH 3T3 | FIBROBLAST | 1.0 | 3.0 | 3.1 | 3.3 |
| HEP G2 | LIVER | 1.0 | 5.2 | 6.5 | 4.3 |
| SHM | MYOMETRIUM | 1.0 | 1.9 | 2.2 | 2.0 |
| SY5Y | NEUROBLASTOMA | 1.0 | 2.3 | 2.1 | 2.1 |
| CEF | FIBROBLAST | 1.0 | 3.2 | 2.2 | 2.5 |
| CV-1 | KIDNEY | 1.0 | 3.1 | 5.3 | 2.2 |
| MDA453 | BREAST | 1.0 | 1.1 | 2.1 | 8.4 |
| CHO | OVARY | 1.0 | 1.8 | 2.2 | 3.3 |
| F9 (*2) | TERATOCARCINOMA | 3.2 | 2.4 | 1.1 | 1.4 |

*1. Activities were determined in triplicate transfections. Activities were normalized to an actin-HCG internal control and expressed relative to values obtained from the collagenase promoter in cells that were not transfected with ER or treated with hormone (see Materials and Methods). Standard deviations (not shown) were less than 20%.

*2 Unliganded ER increased the basal activity of the collagenase promoter.

Table 1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AP1
517
+63
COLL73
AP1
-73
+63
COLL60
-60
+63
COLL517mAP1
M
517
+63
Estradiol
Tamoxifen
ICI
No Hormone
0 2 4 6 8 10 12 14 16 18
CAT ACTIVITY
B)
AP1-TK-CAT
AP1
TK
-109
+45
TK-CAT
TK
-109
+45
0 1 2 3 4 5 6 7
CAT ACTIVITY
C)
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+63
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+45
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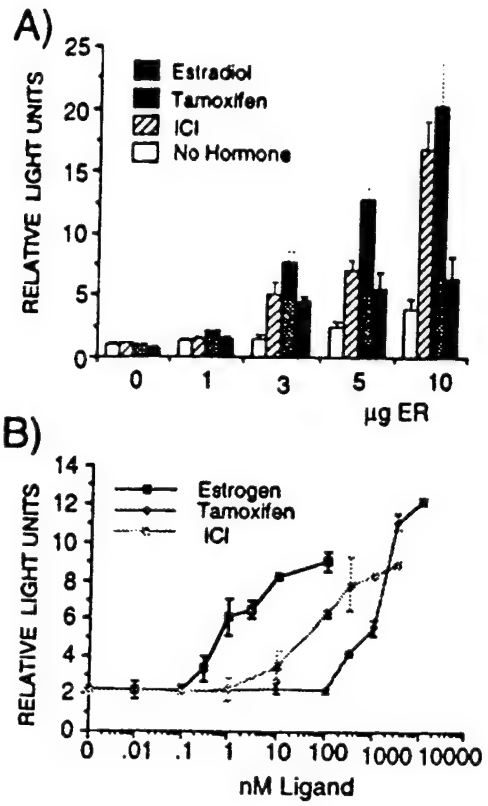
CAT ACTIVITY

Figure 1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10
5
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Tamoxifen
ICI
No Hormone
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3
5
10
µg ER
B)
RELATIVE LIGHT UNITS
14
12
10
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6
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1000
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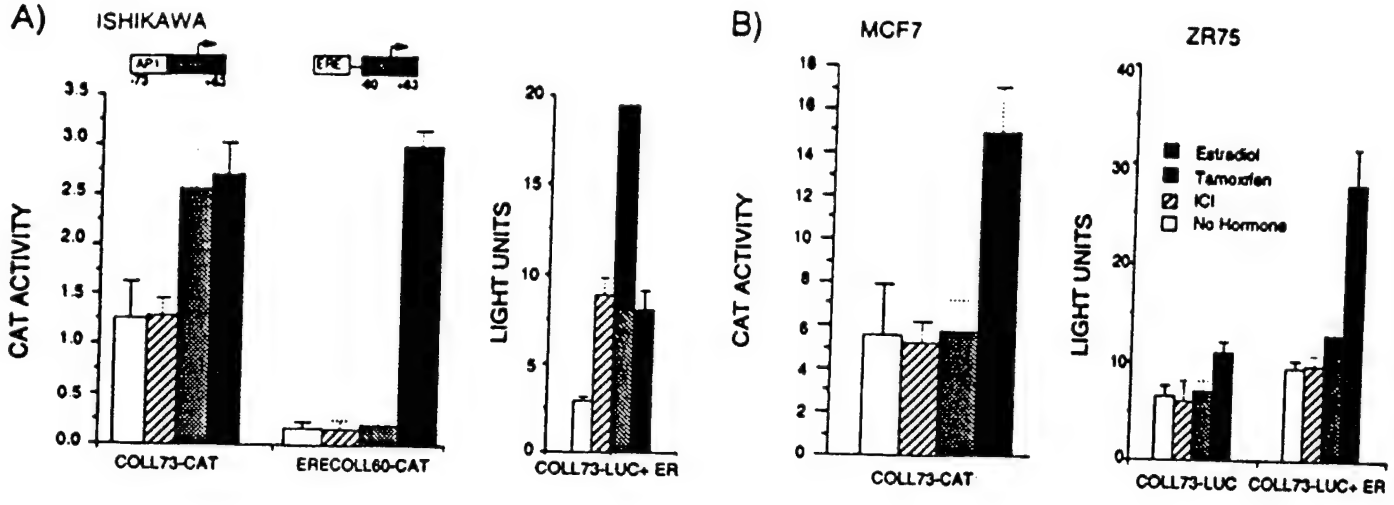
Figure 2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AP1
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COLL73-LUC+ ER
B)
MCF7
CAT ACTIVITY
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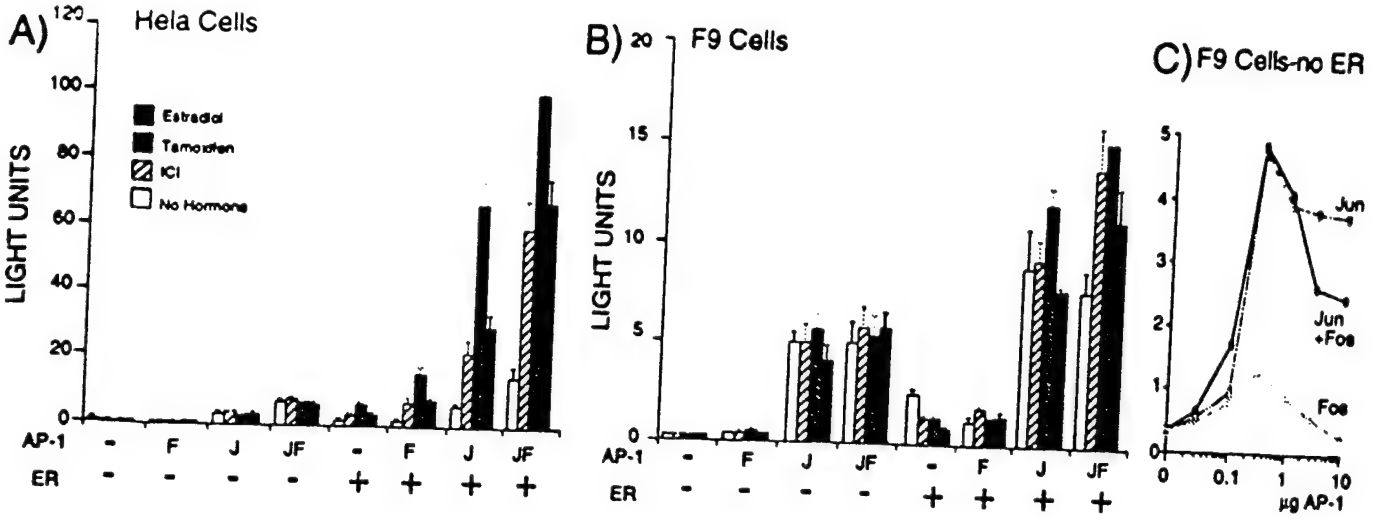
Figure 3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Tamoxifen
ICI
No Hormone
AP-1 - F J JF - F J JF
ER - - - - + + + +
B)
F9 Cells
LIGHT UNITS
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AP-1 - F J JF - F J JF
ER - - - - + + + +
C)
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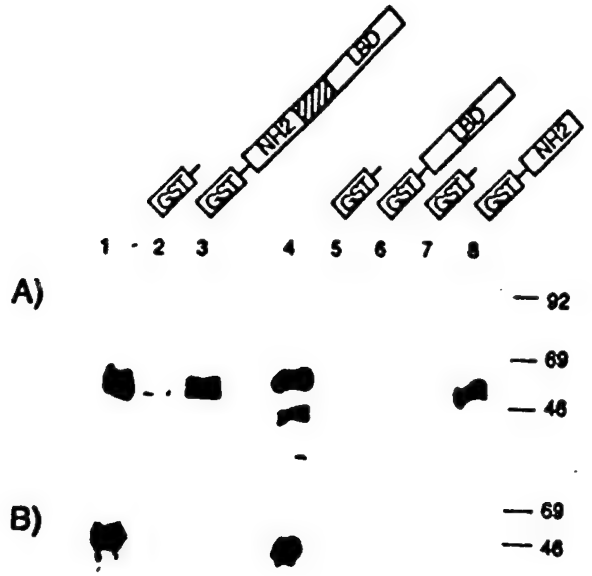
µg AP-1

Figure 4

Figure 5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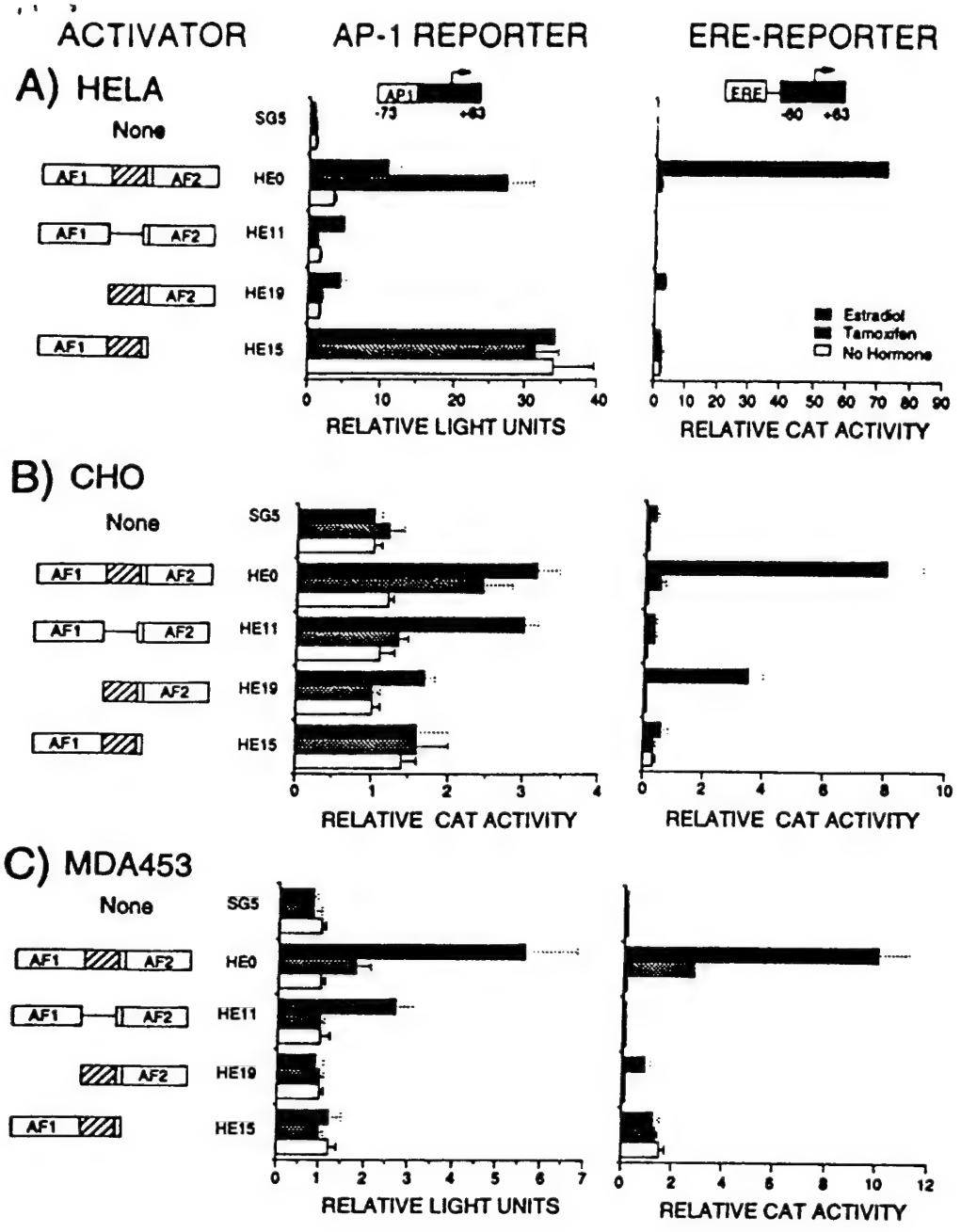
46
B)
69
46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+63
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+63
Estradiol
Tamoxifen
No Hormone
RELATIVE LIGHT UNITS
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C) MDA453
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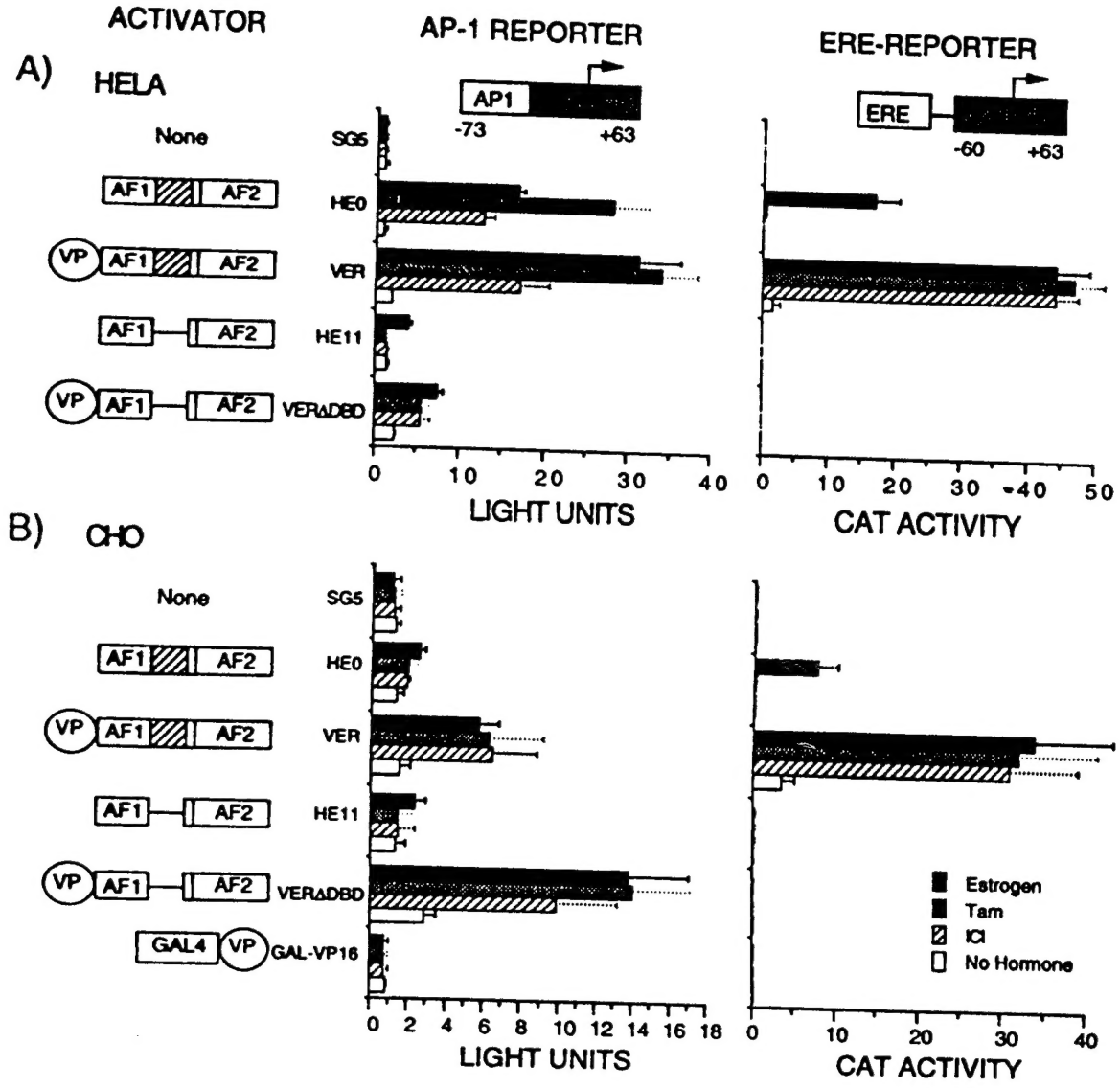
RELATIVE CAT ACTIVITY

Figure 6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-73
+63
ERE
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+63
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LIGHT UNITS
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CAT ACTIVITY
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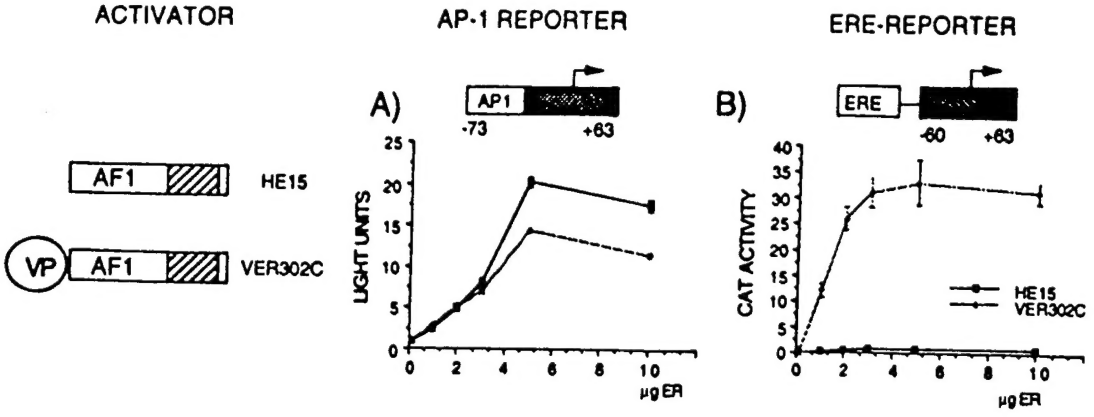
Estrogen
Tam
ICI
No Hormone

Figure 7

Figure 8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A)
AP1
-73
+63
LIGHT UNITS
μg ER
B)
ERE
-60
+63
CAT ACTIVITY
HE15
VER302C
μg ER

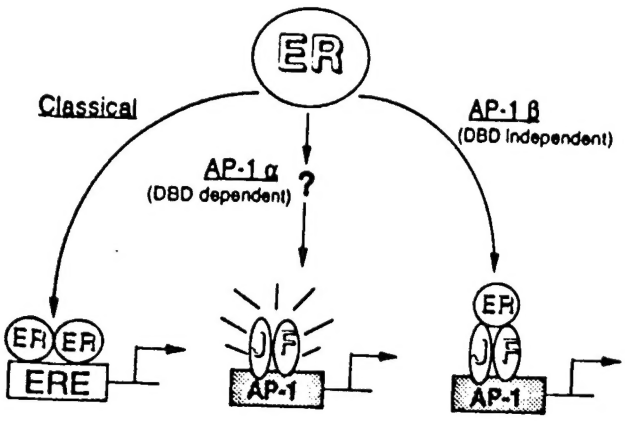
ER
Classical
AP-1 α
(DBD dependent)
?
AP-1 β
(DBD independent)
ER
ER
ERE
J
F
AP-1
ER
J
F
AP-1

Figure 9

# Information requested in the cover letter.

Bibliography of publications:

Webb P, Lopez GL, Uht RM, Kushner PJ. Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell specific estrogen-like effects of antiestrogens. Molecular Endocrinology. 9:443-456. 1995.